Gender dependent association of the $-463G/A$
myeloperoxidase polymorphism with impaired
vasodilation in CAD patients

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ABSTRACT

BACKGROUND Myeloperoxidase (MPO) is an oxidant generating enzyme implicated in coronary artery disease (CAD) through its ability to consume the vasodilator nitric oxide and oxidize LDL, and by recent findings that higher levels of MPO activity correlate with risk for CAD. In addition, a functional −463G/A MPO promoter polymorphism has been linked to risk for CAD.

METHODS In this report, we assay for association of the MPO promoter polymorphism with clinical aspects in 283 cases from the Netherlands referred for diagnostic coronary angiography due to angina pectoris.

RESULTS In females, but not in males, the higher expressing GG genotype was associated with impaired dilative response to nitro-glycerine: the percent dilation of cardiac vessels relative to baseline was 12.4 % for GG versus 16.2 % for GA/AA genotypes (P = 0.027). Also, the GG genotype was associated with increased incidence of angina pectoris in females, but not males, who were past smokers (P = 0.046). In males, there was association of GA/AA genotypes with increased frequency of hypercholesterolemia (55.6% of GA/AA males versus 40.1% of GG males, P = 0.048).

CONCLUSIONS These findings suggest gender-dependent effects of MPO gene expression on vasodilative responses and serum cholesterol levels. Possible mechanisms are discussed.
INTRODUCTION

Myeloperoxidase (MPO) is secreted by reactive neutrophils and monocyte-macrophages at sites of inflammation, where it catalyses a reaction between hydrogen peroxide and chloride to produce the potent oxidant, hypochlorous acid (HOCl)(1). MPO generated oxidants function as antimicrobial agents, yet can also inadvertently damage bystander cells, such that MPO has been implicated as a contributory agent in a number of inflammatory or autoimmune diseases (2-18).

Atherosclerosis and coronary artery disease are chronic inflammatory states with early stages marked by fatty streaks in vessel walls, formed by infiltrating monocyte-derived macrophages and T cells, along with deposits of extracellular lipid (19;20). Inflammatory responses cause the endothelium to become more adhesive and permeable, allowing monocyte-macrophages to infiltrate the subendothelial intima, and phagocytise accumulating LDL particles. Oxidative modification of LDL greatly increases its rate of uptake by macrophages through scavenger receptors (21), leading to formation of lipid-laden foam cells, a hallmark of early atherosclerotic lesions. The eventual death of foam cells at the site releases oxidized lipid to an accumulating pool at the necrotic core of the lesion, whose eventual rupture results in myocardial infarction.

Several lines of evidence suggest MPO plays a role in atherosclerosis and coronary artery disease (CAD). Levels of MPO in neutrophils or serum are significantly higher in patients with established CAD (22). Earlier studies link MPO levels to endothelial dysfunction (ED), an early indicator of CAD or atherosclerosis (23;24). ED is detected as impaired vasodilative responses of coronary arteries to acetylcholine, an inducer of eNOS, or nitro-glycerine, as an endothelium-independent source of the vasodilator, nitric oxide (NO). MPO is suspected to contribute to ED by reducing bioavailability of NO, due to direct consumption by the MPO enzyme or by its by-product substrate radicals (25;26). MPO has been found to effectively reduce NO levels in blood plasma even though this medium contains many other physiological competitive substrates of MPO (26).

MPO is detected in macrophage foam cells at atherosclerotic lesions (27), colocalizing with MPO-dependent oxidation products such as 3-chlorotyrosine (28;29). These observations place MPO at lesions, but do not prove the enzyme contributes to pathology, as opposed to being a marker for reactive monocyte-macrophages at the site. A functional promoter polymorphism provides a means to assay for a causative role for MPO in a disease state (3). The –463 G/A polymorphism is situated within an Alu-encoded hormone response element (30) and creates an SP1 site in the G allele promoter (3), and an estrogen receptor binding site in the A promoter (4). The GG genotype is predominant, present in 60-66% of the
population (5;14;17), and supports two to three fold higher expression of MPO mRNA and protein than GA/AA genotypes (5). This higher expressing GG genotype has been associated with increased incidence of coronary artery disease (6), as well as myeloid leukaemia (3;5;31), multiple sclerosis (7), Alzheimer’s disease (8), MPO-ANCA vasculitis (15), chronic granulomatous disease (9), lung cancer (10;14), Helicobacter infections (12) and periodontal disease (17). The GA/AA genotypes are associated with increased risk for certain disease aspects, such as relapse frequency in MPO-ANCA vasculitis (15), or degree of hepatic fibrosis in HCV-induced hepatitis (16). The GA/AA genotypes have also been associated with risk in older Finnish males for Alzheimer’s (4) or lung cancer (13), and with risk of severe infections following transplant of GA/AA bone marrow (18). Gender differences in MPO genotype association have been observed in some studies (4;7;8;12;14-17), but not others (6;9-11). GG genotype was found to be a female specific risk factor in periodontal disease (17) and MPO-ANCA vasculitis (15), and a male specific risk factor in lung cancer (14). These findings suggest gender differences in regulation of the MPO alleles.

In the present study, we correlate MPO genotypes with clinical aspects of coronary artery disease in 283 cases from The Netherlands. Findings indicate a gender-dependent association of MPO genotype with vasodilative responses and hypercholesterolemia.

**MATERIAL AND METHODS**

**Study population.** Data was collected for 283 patients (180 males and 103 females) from the Netherlands who had experienced angina pectoris and been referred for diagnostic coronary angiography with concomitant intracoronary acetylcholine and nitro-glycerine infusion. Excluded were patients with unstable angina, recent myocardial infarction, valvular heart disease requiring surgical intervention, clinical evidence of heart failure, a history of previous coronary angioplasty, coronary bypass surgery, or any serious disease that may interfere with the follow-up. Other clinical data analysed for these cases included age, sex, smoking history and serum lipid profile. ApoE genotype was determined for 223 cases. The MPO genotype was determined for the 283 cases, along with 129 ethnically matched healthy controls with no history of heart disease. This study was approved by an institutional review committee and the subjects gave informed consent.

**Quantitative Coronary Angiography.** Quantitative Coronary Angiography (QCA) was performed by a previously described and validated automatic contour detection technique (CMS, Medis Co., Nuenen, the Netherlands.
End-diastolic frames of the non-stenotic proximal segment of the LAD and LCX were selected for QCA. User interaction was limited to the definition of the start and end points of the coronary segment to be analysed. Easily identifiable side branches were used as anatomic landmarks to allow the analysis of the same segments in successive angiograms. The length of the analysed segment was always within a range of 10% from baseline segment length. In case of overlap with other branches, automatic contour detection was manually edited by the independent analyst. Segment diameter was determined in millimetres. The coronary vasomotor responses were the changes in mean LAD and LCX coronary diameter, in response to maximal concentration of acetylcholine and nitro-glycerine respectively, expressed as the percentage of the mean baseline diameter. A negative response represents a vasoconstriction.

Genotyping of MPO. Genomic DNA was extracted from white blood cells by standard procedures. Polymerase chain reaction (PCR) was performed with 50-100 ng of genomic DNA with 0.2 μg of each primer in a 25 μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 200 μM nucleotides, and 1 unit of Taq polymerase (Perkin Elmer Cetus). Primers (Genset, Inc.) were 5' CGGTATAGGCACACAATGGTGA 3' and 5' GCAATGGTTCAAGCGATTCTTC 3'. The cycling conditions were 95°C for 6 minutes, followed by 35 cycles at 95°C for 30 sec, 62°C –0.3°C per cycle, for 1 min, and 72°C for 30 sec. The 350 bp reaction product was digested overnight with 10 units of AciI restriction enzyme, which cuts at –463 of the G allele and a second common site to give rise to fragments of 169, 120, and 61 bp. The A allele gives rise to fragments of 289 and 61 bp.

Statistical methods. Statistics were determined using Statview or Mathematica software (SAS Institute, Cary, NC). The relationship between MPO genotypes and disease parameters was assessed by ANOVA, or 2 x 2 contingency matrices. Analyses of gender differences are presented without a correction for multiple statistical significance tests based on previously established gender differences in MPO genotype association with disease states (4;7;8;12;14-17). A p value below 0.05 is considered significant, while p values between 0.05 and 0.08 are considered noteworthy trends.

RESULTS

MPO genotype is not associated overall with incidence in a Netherlands cohort referred for angina pectoris. MPO genotypes were determined for 283 patients who were referred for coronary
angiography due to angina pectoris. Overall, the MPO genotype frequencies did not differ between the 283 cases and 129 controls. The genotype frequencies were homozygous GG 63.6 %, heterozygous G/A 33.6 %, and homozygous AA 2.8 %. The control genotype frequencies were 64.3 % GG, 34.1 % GA, and 2.3 % AA. These genotype distributions were in Hardy-Weinberg equilibrium, did not differ between males and females, and were consistent with MPO genotype frequencies previously reported for Caucasian populations (5;11;15;17). Due to the low numbers of AA genotypes, G/A and AA genotypes are combined in analyses in this study as MPO A allele carriers (GA/AA).

In females, GG genotype is associated with impaired vasodilation in response to infusion of nitro-glycerine. Endothelial dysfunction is assayed as the dilative response of coronary vessels to infusion of nitro-glycerine or acetylcholine. Acetylcholine induces endothelial nitric oxide synthase (eNOS) to produce NO, which signals underlying smooth muscle cells to relax, allowing vessel dilation. Nitro-glycerine infusion measures the endothelium-independent dilative response of vessels by providing an independent source of NO. In this study, the GG genotype was associated with impaired vasodilation in response to nitro-glycerine in females. The vasomotor responses were scored as the percent change from the baseline diameter. Following nitro-glycerine administration, the mean percent increase in vessel diameter (± SEM) was 12.4 (± 1) % for GG females and 16.2 (± 1.3) % for GA/AA females; P = 0.027. In contrast, GG genotype did not correlate with impaired vasodilation in male cases; Percent increase in vessel diameter was 14 (± 1) % for GG males compared to 12.7 (± 1) % for GA/AA males; P = 0.3 (Table 1).
MPO POLYMORPHISM ASSOCIATED WITH IMPAIRED VASODILATION

<table>
<thead>
<tr>
<th>Sex</th>
<th>GG</th>
<th>GA/AA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>Female</td>
<td>GA/AA</td>
<td>P value</td>
</tr>
<tr>
<td>Male</td>
<td>63 (61%)</td>
<td>40 (39%)</td>
<td>ns</td>
</tr>
<tr>
<td>Male</td>
<td>117 (65%)</td>
<td>63 (35%)</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>Female</td>
<td>62 ± 1</td>
<td>ns</td>
</tr>
<tr>
<td>Male</td>
<td>56 ± 1</td>
<td>57 ± 2</td>
<td></td>
</tr>
<tr>
<td>Response to NTG</td>
<td>Female</td>
<td>12.4 ± 1.0</td>
<td>16.2 ± 1.3</td>
</tr>
<tr>
<td>Male</td>
<td>14.0 ± 1.0</td>
<td>12.7 ± 1.0</td>
<td>0.30</td>
</tr>
<tr>
<td>Response to Ach</td>
<td>Female</td>
<td>-7.7 ± 0.8</td>
<td>-10.6 ± 1.7</td>
</tr>
<tr>
<td>Male</td>
<td>-11.0 ± 1.2</td>
<td>-12.3 ± 1.6</td>
<td>0.46</td>
</tr>
<tr>
<td>Hchol (%)</td>
<td>Female</td>
<td>34 (54 %)</td>
<td>20 (50 %)</td>
</tr>
<tr>
<td>Male</td>
<td>47 (40 %)</td>
<td>35 (56 %)</td>
<td>0.048 S</td>
</tr>
<tr>
<td>ApoE4 yes/no (%)</td>
<td>Female</td>
<td>19/30 (39 %)</td>
<td>7/21 (25 %)</td>
</tr>
<tr>
<td>Male</td>
<td>30/61 (33 %)</td>
<td>17/38 (31 %)</td>
<td>0.86</td>
</tr>
<tr>
<td>Smoking history N (%)</td>
<td>Female</td>
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<td>20 (50)</td>
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<tr>
<td>Past smoker (&gt;3 mo)</td>
<td>28 (44)</td>
<td>10 (25)</td>
<td>0.046 S</td>
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<tr>
<td>Current</td>
<td>10 (16)</td>
<td>10 (25)</td>
<td>ns</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>39 (33)</td>
<td>19 (30)</td>
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Table 1. Clinical characteristics and MPO genotype (mean ± SEM) of 283 patients referred for diagnostic coronary angiography according to -463 G/A MPO promoter genotype. NTG = nitro-glycerine, Ach = acetylcholine, Hchol = hypercholesterolemia (total cholesterol > 6.5 mM or on lipid lowering drugs). ApoE was determined for 223/283 cases. ANOVA derived P value.

MPO genotype associates with smoking related risk in females.
Tobacco smoking is a risk factor for CAD and atherosclerosis. The GA/AA genotypes have been associated with reduced smoking-related risk for lung cancer (10;14) and periodontal disease (17). This led us to assay for correlations between smoking and MPO genotype in the present study. Females with GG genotype who were past smokers were over represented (Table 1): Forty four percent of female GG cases were past smokers, as compared to 25 % of GA/AA females (P = 0.046). In contrast, there was no correlation between smoking history and MPO genotype in males: 53 % of GG male cases were past smokers, compared to 51 % of GA/AA males (P = 0.78).

The GA/AA genotype is associated with hypercholesterolemia in males. Endothelial dysfunction often correlates with hypercholesterolemia, a risk factor for atherosclerosis (32). The MPO GA/AA genotypes were recently linked to increased levels of total
cholesterol, LDL cholesterol, and triglycerides in a healthy population (33), leading us to assay for associations between hypercholesterolemia and MPO genotype in the present study. Hypercholesterolemia was here defined as being on cholesterol lowering medications for at least 3 months or having total cholesterol levels greater than 6.5 mmol/L (Table 1). The GA/AA genotype was found to be associated with hypercholesterolemia in males: 55.6% of GA/AA males were classified as hypercholesterolemic as compared to 40.1% of GG males (P = 0.048). In contrast, MPO genotype in females did not associate with hypercholesterolemia: 53.9% of GG and 50% of GA/AA females were hypercholesterolemic (P = 0.69). We also looked for associations between MPO genotype and presence of ApoE4 allele, based on earlier findings that ApoE4 is linked to risk for CAD (34), and our prior findings of interaction between MPO GA/AA and ApoE4 genotypes in males with Alzheimer’s disease (4). There was a potential trend for interaction between GG and ApoE4 genotypes in females, which did not reach significance (Table 1). Similarly there was a trend towards association of GA/AA genotype and response to acetylcholine in females, which did not reach significance (Table 1).

**DISCUSSION**

These findings suggest that the −463G/A promoter polymorphism associates with two risk indicators for coronary artery disease, impaired vasodilative responses and serum cholesterol levels. The patients in this study had been referred for diagnostic coronary angiography due to angina pectoris, a symptom of inadequate oxygenation of heart tissue, potentially reflecting impaired vasodilation. Infusion of nitro-glycerine as NO donor resulted in less vasodilation in GG females than GA/AA females. Based on prior evidence that GG is the higher expressing genotype, this implies that higher levels of MPO correlate with impaired vasodilation. A likely explanation, based on recent studies, is that MPO and its by-products consume nitric oxide, effectively reducing levels of this vasodilator. Depletion of NO could be due to reaction with MPO free in serum, or MPO may be secreted locally by reactive monocytes adhering to inflamed vascular endothelial cells, or by monocyte-macrophages invading the subendothelial intima.

Females (but not males) who were past smokers and GG genotype were over represented in this study group, relative to GA/AA females. This suggests that smoking enhances the deleterious effects of GG genotype, possibly due to selective transcriptional activation of the G allele in leukocytes activated by smoking (35). Consistent with these observations, a recent study of 1083 patients and 2065 controls showed that GA/AA
genotype reduced the smoking related increase in risk for severe periodontal disease in females (OR = 0.5), but not males (17). Thus, past smoking exacerbates risk in GG females for angina pectoris or periodontal disease.

A gender difference was observed in MPO genotype association with both vasodilative responses and cholesterol levels. Males, but not females, with GA/AA genotype had higher incidence of hypercholesterolemia. Some prior studies (4;7;8;12;14-17), but not others (6;9-11), have noted gender differences in MPO genotype association with disease. The GG genotype was found to be a female-specific risk factor for MPO-ANCA vasculitis (15), periodontal disease (17), multiple sclerosis (7) and Alzheimer’s (4;8), while GG has been found to be a male risk factor in lung cancer (14) and Helicobacter infections (12). These findings suggest the two alleles are regulated differently in males and females, possibly through estrogen receptor binding to the –463A site (4). Several lines of evidence suggest estrogen can impact MPO activities. Estrogen is reported to enhance MPO activity in isolated neutrophils (36). The level of MPO in neutrophils is higher in females than males, and fluctuates with serum estrogen levels (37;38), while serum MPO levels is lower in females than males (33), perhaps reflecting estrogen effects on degranulation. Consistent with that hypothesis, treatment of post surgical males with 17β-estradiol reduced the serum levels of MPO (39). Estrogen appears to modulate myeloid cell numbers, in that the numbers of monocytes increase at menopause, and decline following estrogen replacement therapy (40). Thus estrogen has been found to alter MPO activity levels by influencing gene expression, monocyte number, or the degree of release of vesicular MPO through degranulation. The latter mechanism could alter levels of MPO in serum which could impact the levels of vasodilator NO or serum cholesterol.

Prior studies have suggested that MPO affects vasodilative responses by altering NO concentrations. MPO has been shown to react with, thereby consuming NO (25). Recent findings using rodent models of inflammation and human serum suggest MPO modulates NO signalling and vasodilative responses, perhaps due to catalytic consumption of NO by MPO generated substrate radicals (26). The latter study suggests that serum MPO, secreted by activated leukocytes, permeates the vascular endothelial cells and subendothelial matrix. In other studies, incubation of rat aortic rings with chlorinated arginine was shown to inhibit acetylcholine-induced vasorelaxation, apparently due to chlorination by HOCl of L-arginine, creating an inactive substrate for eNOS, thereby inhibiting NO production (41). In other studies, neutrophil adherence to endothelial cells inhibited generation of NO (42), and infusion of HOCl into guinea pig coronary vessels reduced blood flow and blocked the dilative response to acetylcholine (43). In rat models of traumatic shock, increased levels of
MPO were found to correlate with severe endothelial dysfunction (44;45). Thus, MPO secreted into the serum, or released locally by monocytes or neutrophils adhering to the vascular endothelium, or released by invading macrophages in the subendothelial intima, could reduce local NO levels, impairing vasodilative responses. The findings presented here are consistent with these prior studies, indicating that the higher expressing GG genotype is associated with impaired vasodilative response to nitroglycerine, suggesting MPO consumes nitro-glycerine generated NO. In an earlier study using French Canadian patients, the GG genotype was associated with increased incidence of CAD (6). The present study did not detect a difference in MPO genotype frequencies in patients versus controls. A key difference between these studies is the selection criteria. This study selected cases referred for diagnostic coronary angiography due to angina pectoris, while the French Canadian study included patients with angiographically proven plaques in coronary arteries with stenosis obstructing the lumen by at least 30% (6). The GG genotype may be associated with increased incidence of more advanced coronary disease, but not angina pectoris. A second distinction between the two studies is the genetic background of the patient population.

The association of GA/AA genotypes with hypercholesterolemia in males is consistent with a recent report linking GA/AA genotypes to higher levels of total cholesterol, LDL cholesterol, and triglycerides in a healthy population (33). There is prior evidence linking MPO to cholesterol metabolism or transport. MPO dependent tyrosyl radical modification of HDL was found to enhance cholesterol efflux from macrophages (46). Also, MPO-oxidized LDL has been found to inhibit lecithin-cholesterol acetyltransferase, an enzyme required for HDL maturation and the reverse cholesterol transport pathway (47). Third, oxidation of LDL by MPO promotes its uptake by macrophage foam cells (21). While these observations suggest ways in which MPO could alter cholesterol levels, the mechanism has yet to be elucidated.

The association of GA/AA genotype with hypercholesterolemia in males may provide an explanation for previous findings of early mortality for Finnish GA/AA males (4;13). The AA genotype is normally present in 5% of the Finnish subpopulation, yet AA genotypes were absent from a cohort of 159 elderly Finnish males, including Alzheimer’s cases and controls (4). A study of lung cancer cases and controls similarly found reduced frequency of GA/AA genotypes in aging Finnish males (13). Hypercholesterolemia leading to CAD could underlie this selective mortality. Interestingly, the GA genotype was also associated with increased risk for Alzheimer’s disease in Finnish males (4), suggesting that the MPO A allele enhances risk for aging males in diseases involving MPO.
In summary, the findings of this preliminary study suggest that the −463 G/A MPO polymorphism associates with vasodilative responses and hypercholesterolemia in patients with angina pectoris. The association of the higher expressing GG genotype with impaired vasodilative response to nitro-glycerine suggests that MPO released to serum may reduce NO levels generated by nitro-glycerine. This hypothesis is consistent with recent findings that MPO in serum is able to significantly reduce NO levels (26), and that the GG genotype (6) and high MPO protein levels (22) associate with risk for CAD. Thus one interpretation of our findings is that GG genotype, in patients with angina pectoris, correlates with higher serum levels of MPO, reducing NO levels thereby inhibiting vasodilation in response to infusion of nitro-glycerine, and presumably inhibiting vasodilation to endogenous NO inducers, contributing to angina pectoris.

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